

# Amino-Terminal Form of Parathyroid Hormone (PTH) with Immunologic Similarities to hPTH(1–84) Is Overproduced in Primary and Secondary Hyperparathyroidism

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**Background:** To separate non-(1–84)parathyroid hormone [non-(1–84)PTH] from PTH(1–84), we developed new HPLC gradients and observed that the peak coeluting with hPTH(1–84) could be separated into two entities recognized by a cyclase-activating PTH (CA-PTH) assay that reacts with the first four amino acids of the PTH structure.

**Methods:** Sera from six healthy individuals and five patients with primary hyperparathyroidism, and eight pools of sera from patients in renal failure were fractionated by HPLC. A total (T)-PTH assay reacting with the (15–20) region, the CA-PTH assay, and a COOH-terminal (C)-PTH assay with a (65–84) structure requirement were used to measure basal and fractionated PTH values.

**Results:** T-PTH was higher than CA-PTH in all healthy controls [mean (SD), 3.13 (0.37) vs 2.29 (0.33) pmol/L;  $P < 0.01$ ] and in renal failure patients [47 (35.1) vs 33.4 (26.1) pmol/L;  $P < 0.01$ ]. By contrast, CA-PTH concentrations were similar to or higher than T-PTH in three of five patients with primary hyperparathyroidism [25.7 (26.1) vs 23.1 (24.2) pmol/L; not significant]. The CA-PTH assay reacted with the hPTH(1–84) peak and with a minor peak different from the non-(1–84) peak recognized by the T-PTH assay. This minor peak was not recognized by the T-PTH assay. It represented 8 (2)% of CA-PTH in controls, 25 (23)% in patients with primary

hyperparathyroidism, and 22 (7)% in renal failure patients, assuming equimolar reactivity to hPTH(1–84) in the CA-PTH assay. It was not oxidized hPTH(1–84), which migrated differently on HPLC and reacted similarly in the CA and T-PTH assays.

**Conclusions:** This new molecular form of PTH has structural integrity of the (1–4) region but presumably is modified in the region (15–20), which is usually recognized by the T-PTH assay. Its clinical implications remain to be defined.

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The immunoheterogeneous nature of circulating parathyroid hormone (PTH)<sup>3</sup> has given rise over the past 40 years to the ongoing replacement of PTH assays with increasing specificities. Under normocalcemic conditions, circulating PTH is composed of 20% human PTH(1–84) [hPTH(1–84)], the biologically active form of the hormone on the PTH/PTH<sub>rP</sub> receptor, and of 80% COOH-terminal (C-PTH) fragments, considered until recently to be biologically inactive (1–3). In renal failure, C-PTH fragments accumulate because they are cleared mainly by the kidney in individuals with normal renal function (4). In this particular situation, C-PTH fragments represent >95% of circulating PTH (3). Studies in humans have also demonstrated the existence of smaller C-PTH fragments (5) and, more recently, of larger C-PTH fragments with a partially preserved amino-terminal structure (3, 6–8), called non-(1–84)PTH. The latter fragments were discovered by HPLC analysis of circulating PTH with what was presumed to be “intact” PTH (I-PTH) assays (4, 6–8). Non-

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<sup>3</sup> Nonstandard abbreviations: PTH, parathyroid hormone; hPTH, human parathyroid hormone; C-PTH, carboxyl-terminal parathyroid hormone; I-PTH, intact parathyroid hormone; CA, cyclase-activating; and T-PTH, total parathyroid hormone.

(1-84)PTH accounts for 10% of C-PTH fragments and for 20% of I-PTH immunoreactivity in individuals with normal renal function (4). In renal failure patients, it also accounts for 10% of the C-PTH fragments but for >45% of I-PTH immunoreactivity (4, 7, 8).

The possible importance of C-PTH fragments in PTH biology has been disclosed in recent studies. hPTH(7-84), a surrogate for non-(1-84)PTH, and to a lesser extent, smaller C-PTH fragments have hypocalcemic, hypophosphatemic, and hypophosphaturic effects as demonstrated in a thyroparathyroidectomized rat model. hPTH(7-84) also antagonizes the hypercalcemic influence of hPTH(1-84) and hPTH(1-34) in the same model (9, 10). Furthermore, in vitro, hPTH(7-84) is a potent inhibitor of bone resorption induced by various substances (11), and hPTH(7-84) and hPTH(39-84) are inhibitors of vitamin D-induced osteoclastogenesis (11). These biological effects of C-PTH fragments are independent of the type 1 PTH/PTHrP receptor and are exerted through a C-PTH receptor (10, 11).

In our efforts to purify and sequence non-(1-84)PTH, we modified HPLC acetonitrile gradients to better separate non-(1-84)PTH from hPTH(1-84). In doing so, we discovered a new N-terminal PTH molecular form distinct from PTH(1-84) and non-(1-84)PTH and detected only by the cyclase-activating (CA)-PTH assay, which is specific for the first four amino acids of the PTH structure (12, 13). The importance of this new PTH molecular form in various clinical conditions and its immunologic characterization are described here.

## Materials and Methods

### PARTICIPANTS

Seven healthy individuals and five patients with primary hyperparathyroidism participated in this study.

### EXPERIMENTAL PROTOCOL

Blood was obtained by venipuncture in the research laboratory from healthy individuals and patients with primary hyperparathyroidism. The experimental protocol was approved by the institutional Human Ethics Committee, and all individuals signed an informed consent form. Blood was centrifuged immediately after venipuncture, and the serum was stored at -90 °C until further processing. For renal failure, eight pools were constituted at various PTH concentrations (5.8-86.8 pmol/L). These pools were formed from serum that was left over after routine PTH determinations in renal failure patients. Blood was again centrifuged within a maximum of 4 h, and what was left over was stored at -90 °C until further processing. To satisfy requirements of the institutional Human Ethics Committee, all biochemistry renal failure specimens had to be made anonymous before reaching our laboratory. Although it is possible that pooling may have produced PTH forms not representative of specimens from individuals, we have found that this approach has given results similar to those obtained in single

individuals in the past (3). Indices of phosphocalcic metabolism and basal PTH concentrations were measured in serum or pools of serum either fresh or kept at -90 °C. The same sera were used for HPLC analyses.

### EXPERIMENTAL METHODS

The synthetic PTH peptides hPTH(1-84), hPTH(7-84), hPTH(39-84), hPTH(53-84), hPTH(39-68), hPTH(64-84), and hPTH(69-84) were purchased from BACHEM. Mutated [Tyr<sup>34</sup>]hPTH(19-84) was generously provided by H. Juppner (Massachusetts General Hospital, Boston, MA). Oxidized hPTH(1-84) and hPTH(7-84) were produced by the chloramine-T method without the addition of sodium iodide (14). Total calcium, phosphate, alkaline phosphatase, and creatinine were measured by automated colorimetric methods.

Total PTH (T-PTH) and CA-PTH were quantified with commercial assays provided by Scantibodies Laboratory, Inc. (Santee, CA). The detection limit of the T-PTH assay is reported to be 0.13 pmol/L, and the intraassay CV is 4.8% at 2.15 pmol/L. For the CA-PTH assay, the detection limit is 0.1 pmol/L, and the intraassay CV is 6.2% at 3.2 pmol/L (13). C-PTH was measured by an "in-house" RIA reacting predominantly with C-PTH fragments (1-3). Antiserum C-52, presaturated with a molar excess of hPTH(44-68) to eliminate its midmolecule reactivity, was used at a 1:50 000 dilution with <sup>125</sup>I-[Tyr<sup>52</sup>]hPTH(52-84) as tracer and hPTH(39-84) as the calibrator. The detection limit is 1.3 pmol/L, and the intraassay CV is 3.3% at 50% binding. Assay specificities were studied with the use of various PTH calibrators and also through determinations of assay capacities to recognize circulating PTH molecular forms after HPLC fractionation.

PTH forms from all sera were extracted on Sep-Pak Plus C-18 cartridges (Waters Chromatography Division), as described by Bennett et al. (15). One C-18 cartridge was used for each 3 mL of serum. Up to 15 mL of serum was used for healthy individuals. Samples were eluted from the cartridge with 3 mL of 800 mL/L acetonitrile in 1 g/L trifluoroacetic acid. Acetonitrile was evaporated from the eluate with nitrogen; the residual volume was freeze-dried and then reconstituted in 2 mL of 1 g/L trifluoroacetic acid for HPLC analysis. Each 2-mL sample was loaded on a C<sub>18</sub> μBondapak analytical column (3.9 × 300 mm; Waters) and eluted with a noncontinuous linear gradient of acetonitrile in 1 g/L trifluoroacetic acid. Our original acetonitrile gradient went from 10% to 20% in 5 min, 20% to 40% in 40 min, and 30% to 45% in 15 min. Gradient 2 went from 15% to 23% in 25 min, 23% to 30% in 5 min, and 30% to 36% in 30 min. Gradient 3 went from 15% to 23% in 25 min, 23% to 30% in 5 min, and 30% to 33% in 30 min. Each gradient was delivered at 1.5 mL/min with a Model 2700 Solvent Delivery System (Bio-Rad) (3, 6-8). The 1.5-mL fractions were evaporated, freeze-dried, and reconstituted to 1 mL with 7 g/L bovine serum albumin in water; adequate volumes were then measured in the various PTH assays. Control experiments were

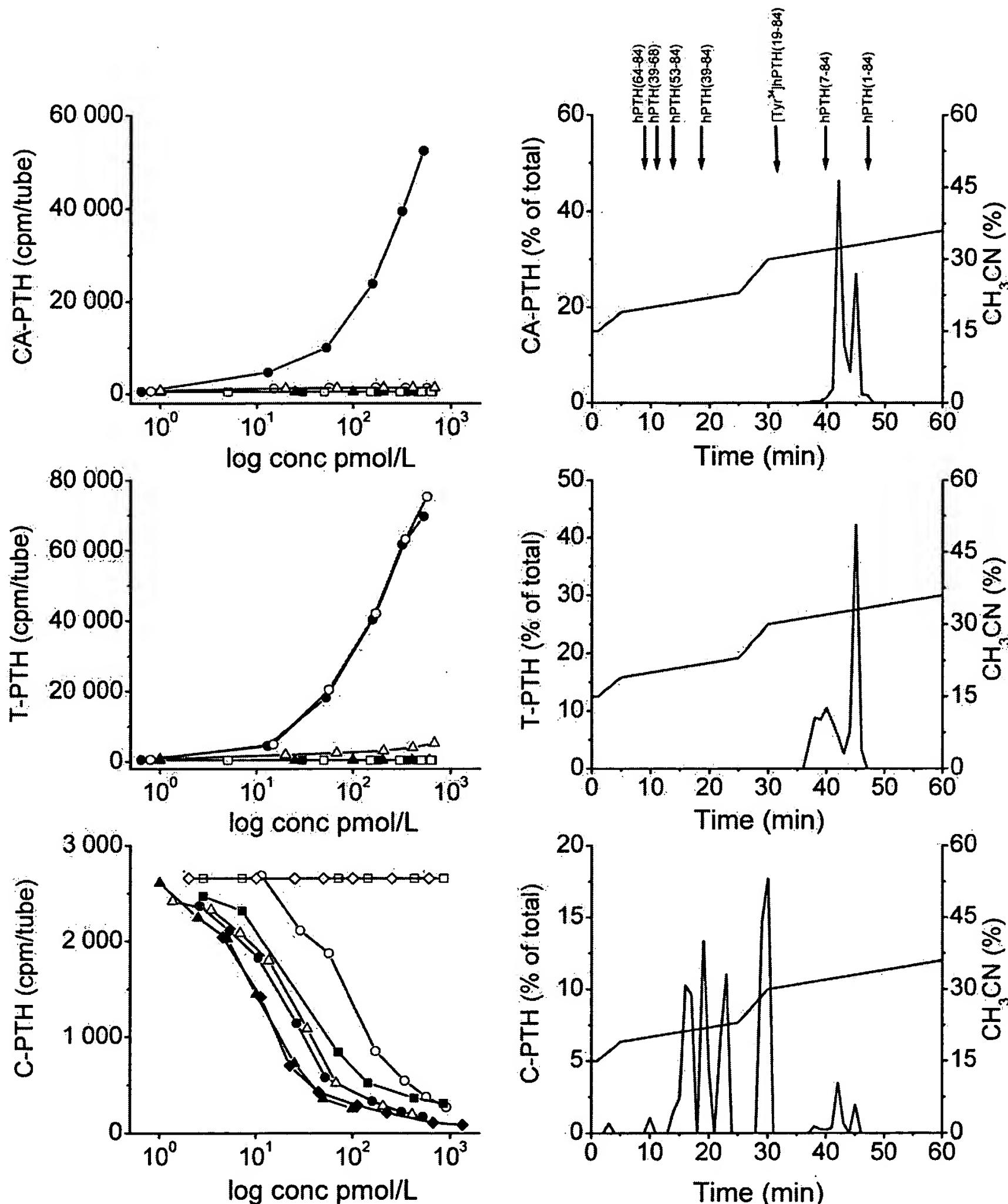


Fig. 1. Characteristics of PTH assays used in this study with PTH calibrators (left panels), and a HPLC profile from a patient with primary hyperparathyroidism (right panels).

Calibrators were as follows: ●, hPTH(1-84); ○, hPTH(7-84); △, [Tyr<sup>34</sup>]hPTH(19-84); ▲, hPTH(39-84); □, hPTH(39-68); ■, hPTH(53-84); ◆, hPTH(64-84); ◇, hPTH(69-84). Acetonitrile gradient 2 (shown in Fig. 3) was used. The CA-PTH assay (top panels) reacted only with hPTH(1-84) and recognized a peak of immunoreactivity coeluting with hPTH(1-84) in position 45 and a new peak of immunoreactivity in position 42-43. The T-PTH assay (middle panels) reacted with hPTH(1-84) and hPTH(7-84) similarly and also recognized hPTH(1-84) in position 45 and non-(1-84)PTH in positions 36-41. The C-PTH assay (bottom panels) reacted mainly with hPTH(39-84) and less with hPTH(1-84) and hPTH(7-84). The C-PTH assay recognized HPLC peaks identified by the two other assays as well as several less hydrophobic peaks (positions 15, 16, 19, 23, and 30).

performed with hPTH(1-84) calibrator added to hypoparathyroidism serum to ensure that PTH degradation did not occur during the various procedures. A single peak of immunoreactivity coeluting with hPTH(1-84) was always detected by the three PTH assays. Immunoreactive PTH recovery by all three PTH assays through all of these procedures was >75% for the 20 patients in HPLC analyses comparing original serum PTH values with the sum of PTH immunoreactivity across all HPLC fractions.

#### DATA ANALYSIS

The results are expressed as the mean (SD). Differences between groups were analyzed by ANOVA, followed by the Student-Newman-Keuls multiple-comparisons test. HPLC profiles were evaluated planimetrically with Origin 4.1 software (Microcal Software).

#### Results

The immunoreactivity of the three PTH assays toward the PTH calibrators and circulating PTH molecular forms separated by HPLC in a patient with primary hyperparathyroidism are shown in Fig. 1. The CA-PTH assay reacted only with the hPTH(1-84) calibrator and not with any of the synthetic C-PTH fragments, including hPTH(7-84). With a more efficient acetonitrile gradient, this assay detected two HPLC peaks. The first, in positions 42-43, migrated in front of the hPTH(1-84) peak (position 45). The T-PTH assay behaved similarly to previously reported I-PTH assays (7). It reacted equimolarly with hPTH(1-84) and hPTH(7-84) but not with any of the smaller C-PTH fragments, including [Tyr<sup>34</sup>]hPTH(19-84), suggesting an epitope in the region (15-20). The assay reacted with hPTH(1-84) in position 45 as well as a broader region in positions 36-41 corresponding to non-(1-84)PTH. It did not react with the peak in position 42-43. The C-PTH assay reacted only with PTH fragments having a structure larger than (65-84) and not with fragment hPTH(69-84). It did not react with the mid-C-terminal fragment hPTH(39-68). Its affinity for various PTH molecules varied, being highest for hPTH(39-84), intermediate for hPTH(1-84), and lowest for hPTH(7-84). It reacted with the molecular forms detected by the CA-PTH and T-PTH assays and also with several less hydrophobic C-PTH fragment peaks migrating in positions 15, 16, 19, 23, and 29.

The biochemical characteristics of the three groups studied are summarized in Table 1. Patients with primary hyperparathyroidism were hypercalcemic with alkaline phosphatase, CA-PTH, T-PTH, and C-PTH concentrations significantly higher than those of healthy individuals, as expected. Pools of sera from renal failure patients also had creatinine, phosphate, alkaline phosphatase, CA-PTH, T-PTH, and C-PTH concentrations significantly higher than those of healthy individuals, again as expected.

The serum HPLC profiles as analyzed by the CA-PTH and T-PTH assays are shown in Fig. 2. Individual HPLC profiles are indicated by the thin lines, whereas the mean

**Table 1. Biochemical characteristics of the groups studied.<sup>a</sup>**

Characteristic	Healthy individuals n	Primary hyperparathyroidism n	Renal failure n
Total calcium, mmol/L	2.36 (0.08)	2.79 (0.19) <sup>b</sup>	2.20 (0.09) <sup>c,e</sup>
Phosphate, mmol/L	1.16 (0.10)	1.00 (0.35)	1.76 (0.14) <sup>b,e</sup>
Alkaline phosphatase, U/L	58.0 (15.3)	93.4 (34.2) <sup>c</sup>	125.9 (52.4) <sup>d</sup>
Creatinine, $\mu$ mol/L	70.6 (11.1)	97.2 (40.7)	835.6 (57.0) <sup>b,e</sup>
T-PTH, pmol/L	3.13 (0.37)	25.7 (26.0) <sup>d</sup>	47.0 (35.1) <sup>b</sup>
CA-PTH, pmol/L	2.29 (0.33)	23.1 (24.2) <sup>d</sup>	33.4 (26.1) <sup>b</sup>
C-PTH, pmol/L	8.03 (1.71)	79.9 (95.8) <sup>d</sup>	157.4 (108.1) <sup>b,f</sup>

<sup>a</sup> Values are means (SD). Statistical analysis by ANOVA followed by the Student-Newman-Keuls test.

<sup>b-d</sup> Healthy individuals vs primary hyperparathyroidism or renal failure:

<sup>b</sup> $P < 0.001$ ; <sup>c</sup> $P < 0.05$ ; <sup>d</sup> $P < 0.01$ .

<sup>e,f</sup> Primary hyperparathyroidism vs renal failure: <sup>e</sup> $P < 0.001$ ; <sup>f</sup> $P < 0.05$ .

HPLC profiles of each group are shown as the thicker line in each panel. The results were qualitatively similar in all groups, but they differed quantitatively from one group to the next. The quantitative planimetric evaluation of HPLC profiles is presented in Table 2. The T-PTH assay reacted with a peak coeluting with the hPTH(1-84) calibrator in position 45 and at least two peaks of non-(1-84)PTH in positions 36-41. Non-(1-84)PTH represented 17 (7)% of the immunoreactivity in controls, 24 (14)% in primary hyperparathyroidism, and 39 (7)% in renal failure, assuming equimolar reactivity of non-(1-84)PTH peaks and hPTH(1-84) in the T-PTH assay. The percentage of non-(1-84)PTH was 2.3 times higher in renal failure patients than in healthy individuals. The CA-PTH assay detected a peak of immunoreactivity coeluting with hPTH(1-84) and a second peak migrating in front of hPTH(1-84) at positions 42 and 43, which was slightly more hydrophobic than non-(1-84)PTH (positions 36-41). Because of the binding specificity of the CA-PTH assay, which requires the presence of the first amino acid (Ser) of PTH to be measured, this new PTH form appears to be N-terminally intact. This new PTH peak corresponded to 8 (2)% of CA-PTH immunoreactivity in controls, 25 (23)% in primary hyperparathyroidism, and 22 (7)% in renal failure, assuming equimolar reactivity of this peak with hPTH(1-84) in the CA-PTH assay. Again, pools of sera from renal failure patients had 2.7 times more of this new PTH molecular form than did healthy individuals.

Fig. 3 shows representations of a detailed immunologic resolution analysis performed on this new molecular form of PTH with the three PTH assays, using various acetonitrile HPLC gradients, in the patients with primary hyperparathyroidism with the highest PTH concentrations. The results are expressed in absolute values to allow direct comparisons between assays. The new PTH molecular form could not be separated from hPTH(1-84) by our original HPLC gradient but was resolved in the two

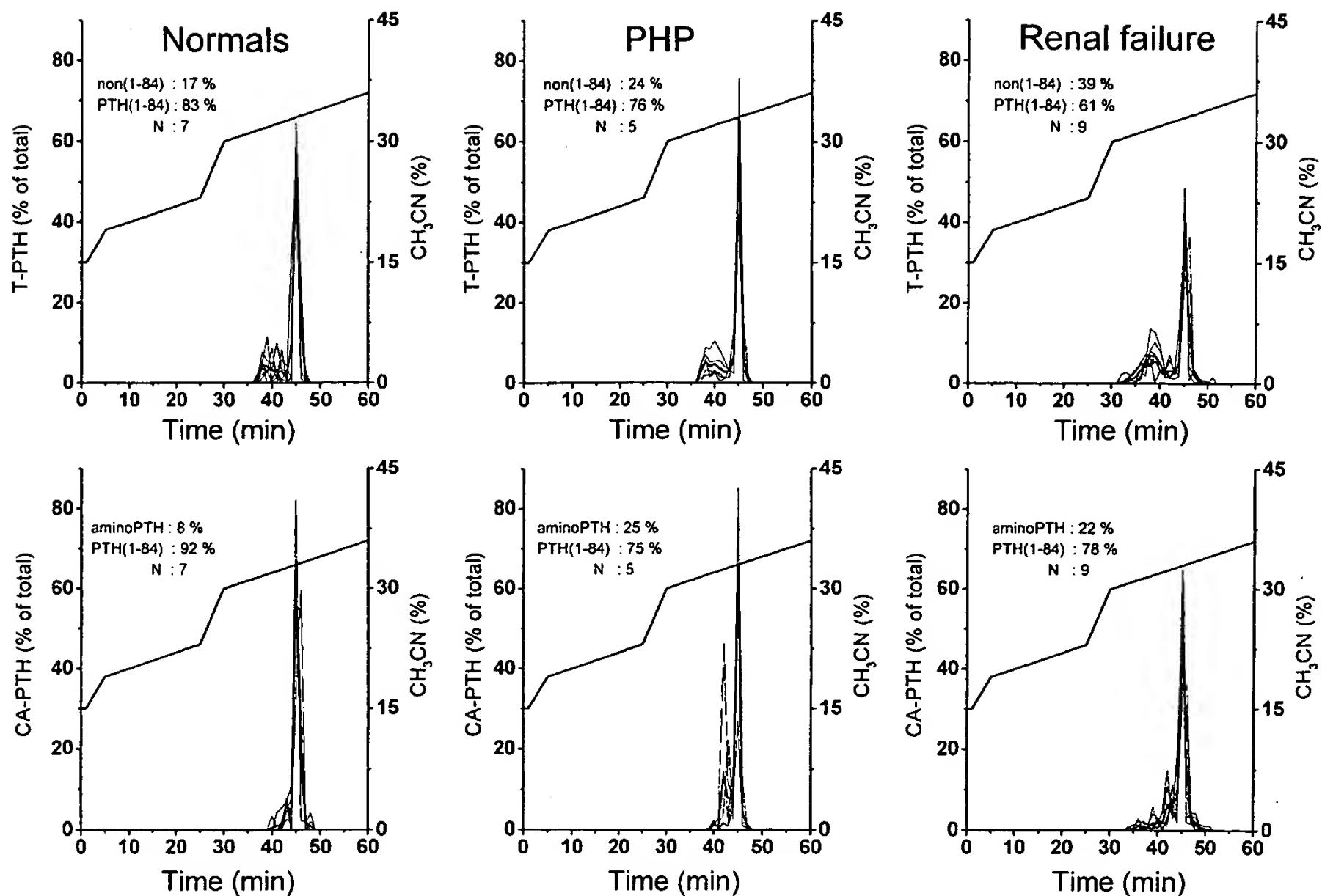


Fig. 2. HPLC profiles of circulating PTH in various populations detected by the T-PTH (top panels) and CA-PTH (bottom panels) assays.

Acetonitrile gradient 2 (shown in Fig. 3) was used. The thin lines represent individual results; the thick lines represent mean results for a group. (Left panels), healthy controls; (middle panels), patients with primary hyperparathyroidism; (right panels), patients with renal failure. The results are qualitatively similar to those shown in Fig. 1, but quantitatively different for each group. The planimetric evaluation of these peaks is summarized in Table 2.

newer gradients. This new form of PTH migrated differently from the non-(1-84)PTH in gradients 2 and 3 and exhibited reactivity in the CA-PTH and C-PTH assays but little, if any, reactivity in the T-PTH assay (gradient 3).

The application of the HPLC planimetric results to the original T-PTH and CA-PTH values, broken down into their components, are also summarized in Table 2. hPTH(1-84) values from the two assays were similar except in controls, where mean CA-PTH hPTH(1-84) was slightly lower. When non-(1-84)PTH was included along with the new N-terminal PTH molecular form, the combined value represented 25% of the PTH measured by the two assays in healthy individuals and 50% in patients with primary hyperparathyroidism or renal failure, again assuming equimolar reactivity of these molecular forms of PTH with hPTH(1-84) in the two PTH assays. In the latter population, the newly discovered molecular form of PTH behaved similarly to non-(1-84)PTH and showed evidence of accumulation with renal failure.

Representative results of a detailed immunologic resolution analysis performed on oxidized hPTH(1-84) and hPTH(7-84) with the CA- and T-PTH assays, using HPLC

acetonitrile gradient 2, are shown in Fig. 4. Oxidized hPTH(1-84) migrated in position 38, differently from hPTH(1-84), which migrated in position 45, and from the newly discovered PTH molecular form, which migrated in positions 42-43. Oxidized PTH reacted equally in the CA- and T-PTH assays, whereas the newly discovered PTH molecular form reacted only in the CA-PTH assay. Oxidized hPTH(7-84) migrated in position 36, differently from hPTH(7-84), which migrated in position 39. Both forms reacted equally in the T-PTH assay but not in the CA-PTH assay.

### Discussion

This study was initiated to obtain more information on a new molecular form of circulating PTH uniquely detected by the CA-PTH assay when more efficient HPLC gradients were used to separate the previously described non-(1-84)PTH from PTH(1-84).

The unique specificities of the three PTH assays, particularly the CA-PTH assay, are of importance in this study because they allowed us to deduce a possible structure for this newly discovered PTH molecular form.

**Table 2. Planimetric evaluation of HPLC profiles applied to T-PTH and CA-PTH values.<sup>a</sup>**

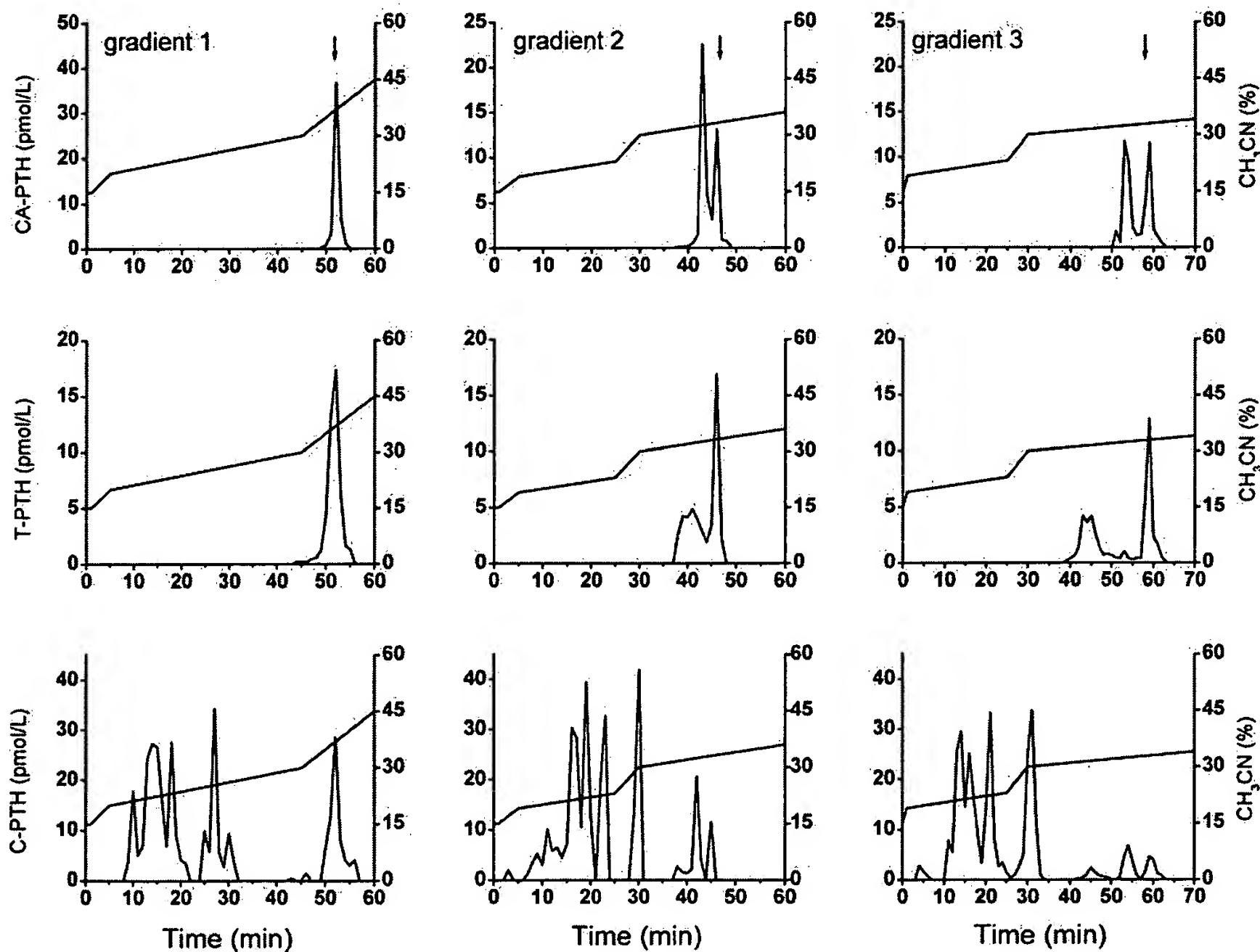
Group	n	T-PTH assay				CA-PTH assay			
		Non-(1-84)PTH		PTH(1-84)		Amino-PTH		PTH(1-84)	
		%	pmol/L	%	pmol/L	%	pmol/L	%	pmol/L
Controls	7	17 (7)	0.55 (0.24)	83 (7)	2.58 (0.31)	8 (2)	0.19 (0.07)	92 (2)	2.10 (0.28) <sup>b</sup>
Primary hyperparathyroidism	5	24 (14) <sup>c</sup>	7.0 (9.9) <sup>d</sup>	76 (14)	18.7 (19.6) <sup>c</sup>	25 (23) <sup>d</sup>	8.8 (15.8) <sup>c</sup>	75 (23)	14.4 (13.5) <sup>c</sup>
Renal failure	8	39 (7) <sup>e</sup>	18.1 (14.7) <sup>c</sup>	61 (7) <sup>c</sup>	28.9 (20.7) <sup>e</sup>	22 (7) <sup>c</sup>	8.5 (9.3) <sup>e</sup>	78 (8)	25.0 (17.8) <sup>e</sup>

<sup>a</sup> Results are means (SD).<sup>b</sup> Statistical analysis by paired Student *t*-test for T-PTH vs CA-PTH: *P* < 0.01.<sup>c-e</sup> Statistical analysis by ANOVA followed by the Student-Newman-Keuls test for controls vs primary hyperparathyroidism or renal failure: <sup>c</sup>*P* < 0.01; <sup>d</sup>*P* < 0.05; <sup>e</sup>*P* < 0.001.

The CA-PTH assay uses a solid-phase (39-84) antibody to capture PTH and a unique (1-4) directed antibody to reveal hPTH(1-84) with a binding dependence on the presence of the first amino acid (12). The CA-PTH assay detects hPTH(1-84) only (12, 13) and a single peak of immunoreactivity coeluting with hPTH(1-84) when cir-

culating PTH is resolved by use of our old HPLC gradient (13). With more efficient gradients, that single hPTH(1-84) peak was separated into two different entities, both with an intact N-terminal structure that included the first amino acid.

The T-PTH assay uses the same capture antibody and

**Fig. 3. Influence of various acetonitrile gradients on the separation of circulating PTH molecular forms by HPLC.**

Serum from a patient with primary hyperparathyroidism and three different PTH assays were used. (Left panels), gradient 1 (the original method); (middle panels), gradient 2; (right panels), gradient 3. The T-PTH assay detected hPTH(1-84) (position 45) and non-(1-84)PTH (positions 36-41). The CA-PTH assay also detected hPTH(1-84) (position 45) and a peak migrating in front of hPTH(1-84) (positions 42 and 43) that was different from non-(1-84)PTH. This peak reacted in the C-PTH assay, whereas non-(1-84)PTH was much less reactive.

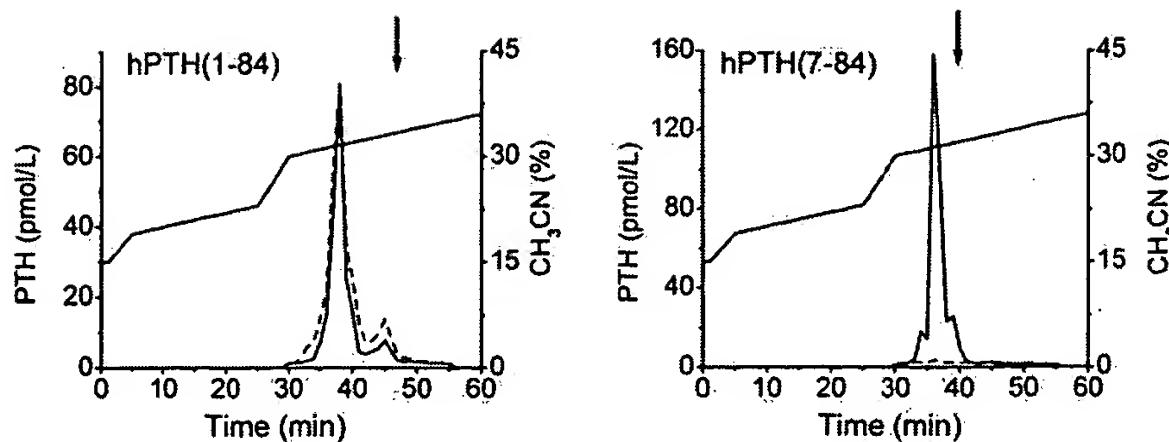


Fig. 4. HPLC profiles of oxidized hPTH(1-84) (left) and hPTH(7-84) (right).

Acetonitrile gradient 2 (shown in Fig. 3) was used. Results with the CA-PTH (dashed line) and T-PTH (solid line) assays. The usual positions of hPTH(1-84) and hPTH(7-84) are indicated by arrows. Oxidized hPTH(1-84) migrated in position 38, whereas oxidized hPTH(7-84) in migrated in position 36. Oxidized hPTH(1-84) reacted equally in the CA-PTH and T-PTH assays, whereas oxidized hPTH(7-84) reacted only in the T-PTH assay.

a detection antibody directed against the (15–20) region of the PTH structure. It detects hPTH(1-84) and hPTH(7-84) equally, but not [ $\text{Tyr}^{34}$ ]hPTH(19–34). It can detect the hPTH(1-84) and the non-(1-84)PTH peaks from HPLC profiles of sera derived from patients with various clinical conditions (13). Its characteristics and behavior are identical to those of other I-PTH assays (7, 8). This T-PTH assay does not detect (or barely detects) the newly discovered molecular form recognized by the CA-PTH assay, suggesting a potential modification in the (15–20) region that might prevent detection.

Finally, the C-PTH assay recognizes hPTH(65–84) but not hPTH(69–84) or mid-C-terminal fragments (3, 6–8), suggesting distal C-terminal specificity for the (65–84) region. The fact that hPTH(7-84) was less reactive in this assay indicates a configuration change specific to this large C-PTH fragment. The C-PTH assay also detected the newly discovered PTH molecular form revealed by the CA-PTH assay. It is difficult to conclude from our results that the C-terminal end of this new molecular form of PTH is necessarily intact. One would need to demonstrate that a fragment larger than (65–84) with its last amino acid removed is nonreactive in this assay to reach that conclusion. This also applies to all C-PTH fragments because their C-terminal ends have never been studied other than immunologically. Nonetheless, these results suggest that we may have discovered a new form of hPTH(1-84) that is not truncated, but possibly modified in the (15–20) region.

To confirm that this new form of PTH was not simply oxidized PTH, with a change on the methionine residue in position 18, we oxidized hPTH(1-84) and hPTH(7-84) by the chloramine-T method and studied their migration on HPLC, using acetonitrile gradient 2. We also studied their immunoreactivity in the CA and T-PTH assays. Oxidized PTH migrated in position 38 in front of hPTH(1-84) and in front of the new PTH molecular form. Furthermore, it was recognized equally by the CA- and T-PTH assays, making it different from the newly discovered form. Even if oxidized PTH migrated in the non-(1-84)PTH region, there was no evidence for oxidized hPTH(1-84) in circulation because there was not reactivity in the CA-PTH assay in that region on the HPLC profiles. This being eliminated, we next considered another possibility. In

1984, Rabbani et al. (16) described a posttranslational modification of the PTH molecule. Phosphorylation in the N-terminal region was demonstrated by use of bovine and human parathyroid glands (16). Phosphorylation of the serine residues in positions 1 and 3 would render immunoreactivity with the CA-PTH assay improbable. Phosphorylation of the serine residue in position 17 could explain its inability to exhibit immunoreactivity in the T-PTH assay and could explain our observation. More studies will be required to test this hypothesis.

The behavior of this new PTH molecular form in the three populations studied is also of interest. Patients with renal failure appear to have higher relative amounts of this new molecular form of PTH compared with that observed in healthy individuals and in some patients with primary hyperparathyroidism. Two patients with primary hyperparathyroidism had a proportion of this new PTH molecular form similar to that in healthy patients, and three others had a much higher amount (17.8–63.3%). In renal failure patients, this new molecular PTH form was increased in the same proportion as reported previously for non-(1-84)PTH (13), suggesting increased production and secretion by hyperfunctional parathyroid glands with decreased renal clearance (17).

Recently, it was suggested that the ratio of (1-84)/non-(1-84)PTH could be a very useful index in identifying renal failure patients with adynamic bone disease from renal failure patients with high/normal bone turnover (18). Such differentiation is very important in the treatment of the secondary hyperparathyroidism of renal failure with vitamin D analogs or by parathyroidectomy. However, the existence of this newly discovered hPTH(1-84) molecular form might have to be taken into account when assessing non-(1-84)PTH by subtraction of the CA-PTH value from the T-PTH value. Direct evaluation of non-(1-84)PTH by the subtraction of CA-PTH from T-PTH might underestimate the total amount of non-(1-84)PTH in renal failure because the CA-PTH values include this newly discovered PTH molecular form, which is not detected by the T-PTH assay. The data presented here demonstrate that the proportion of this new PTH varies in renal failure. Therefore the ratio of (1-84)/non-(1-84)PTH could be affected. Nonetheless, Monier-Fougère et al. (18) were able to demonstrate with

the subtraction method that the ratio of (1-84)/non-(1-84)PTH could differentiate renal failure patients with adynamic bone disease from renal failure patients with normal/high bone turnover disease. This may suggest that the interference of this newly discovered PTH molecular with the calculation of non-(1-84)PTH is not a major problem in these patients. Moreover, although the biological activity of this new form of PTH has not as yet been studied, the clinical utility of PTH measurements with widely accepted PTH assays should not be viewed as impacted by the elucidation of this new molecular form of PTH detected uniquely by the CA-PTH.

In conclusion, we are the first to identify a new species of PTH different from hPTH(1-84) with a preserved amino acid sequence at the NH<sub>2</sub> terminus. This newly discovered form of PTH is immunoreactive and detectable by the CA-PTH assay, but not by the T-PTH assay. We can speculate that if the C-terminal end of this new circulating PTH molecular form is intact, it could interact with both the PTH/PTHrP and C-terminal PTH receptors. The fact that it accumulates in renal failure similarly to other C-PTH fragments could suggest a lack of interaction with the PTH/PTHrP receptor because of a (15-20) posttranslational modification. Finally, if it is shortened at the C-terminal end, it would not interact with the C-PTH receptor (19), and it remains to be seen how it would interact with the PTH/PTHrP receptor. Thus, more studies are required to elucidate the (15-20) and C-terminal structure of this new PTH molecular form. This could be clinically relevant in patients with primary hyperparathyroidism in whom large amounts of this new molecular form could be present, as well as in renal failure patients in whom it accumulates.

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### References

- D'Amour P, Labelle F, Lecavalier L, Plourde V, Harvey D. Influence of serum Ca concentration on circulating molecular forms of PTH in three species. *Am J Physiol* 1986;251:E680-7.
- D'Amour P, Palardy J, Bashali G, Malette LE, Delean A, Lepage R. Modulation of circulating parathyroid hormone immunoheterogeneity in man by ionized calcium concentration. *J Clin Endocrinol Metab* 1992;135:525-32.
- Brossard JH, Cloutier M, Roy L, Lepage R, Gascon-Barré M, D'Amour P. Accumulation of a non-(1-84) molecular form detected by Intact PTH assay in renal failure: importance in the interpretation of PTH values. *J Clin Endocrinol Metab* 1996;81:3923-9.
- D'Amour P, Lazure C, Labelle F. Metabolism of radioiodinated carboxyl-terminal fragments of bovine parathyroid hormone in normal and anephric rats. *Endocrinology* 1985;117:127-34.
- D'Amour P, Labelle F, Wolde-Ghiorghis R, Hamel L. Immunological evidences for the presence of small late carboxyl-terminal fragment(s) of human parathyroid hormone (PTH) in circulation in man. *J Immunoassay* 1989;10:191-205.
- Brossard JH, Whittom S, Lepage R, D'Amour P. Carboxyl-terminal fragments of parathyroid hormone are not secreted preferentially in primary hyperparathyroidism as they are in other causes of hypercalcemia. *J Clin Endocrinol Metab* 1993;137:413-9.
- Lepage R, Roy L, Brossard JH, Rousseau L, Dorais C, Lazure C, et al. A non-(1-84) circulating parathyroid hormone (PTH) fragment interferes significantly with intact PTH commercial assay measurements in uremic samples. *Clin Chem* 1998;44:805-9.
- Brossard JH, Cardinal H, Roy L, Lepage R, Rousseau L, Dorais C, et al. Influence of glomerular filtration rate on intact parathyroid hormone levels in renal failure patients: role of non-(1-84)PTH detected by intact PTH assay. *Clin Chem* 2000;46:697-703.
- Slatopolsky E, Finch J, Clay P, Martin D, Sicard G, Singer G, et al. A novel mechanism for skeletal resistance in uremia. *Kidney Int* 2000;58:753-61.
- Nguyen-Yamamoto L, Rousseau L, Brossard JH, Lepage R, D'Amour P. Synthetic carboxyl-terminal fragments of parathyroid hormone (PTH) decrease ionized calcium concentration in rats by acting on a receptor different from the PTH/PTH-related peptide receptor. *Endocrinology* 2001;142:1386-92.
- Divieti P, John MR, Jüppner H, Bringhurst FR. Human PTH(7-84) inhibits bone resorption in vitro via actions independent of the type 1 PTH/PTHrP receptor. *Endocrinology* 2002;143:171-6.
- John MR, Goodman WG, Gao P, Cantor TL, Salusky IB, Jüppner H. A novel immunoradiometric assay detects full-length human PTH but not amino-terminally truncated fragments: implications for PTH measurements in renal failure. *J Clin Endocrinol Metab* 1999;140:4287-90.
- Gao P, Scheibel S, D'Amour P, John MR, Kao PC, Schmidt-Goyk H, et al. Development of a novel immunoradiometric assay exclusively for biologically active whole parathyroid hormone (1-84): implications for improvement of accurate assessment of parathyroid function. *J Bone Miner Res* 2001;16:605-14.
- D'Amour P, Huet PM. Evidence of two forms of hepatic extraction of parathyroid hormone in dogs in vivo. *Am J Physiol* 1984;246:E249-55.
- Bennett HP, Hudson AM, McMartin C, Purdow GE. Use of octadecylsilica for the extraction and purification of peptides in biological samples. *Biochem J* 1977;168:9-13.
- Rabbani SA, Kremer R, Bennett HP, Goltzman D. Phosphorylation of parathyroid hormone by human and bovine parathyroid glands. *J Biol Chem* 1984;259:2949-55.
- Nguyen-Yamamoto L, Rousseau L, Brossard JH, Lepage R, Gao P, Cantor T, et al. Origin of parathyroid hormone (PTH) fragments detected by intact PTH assays. *Eur J Endocrinol* 2002;147:123-31.
- Monier-Fougère MC, Geng Z, Mawad H, Friedler RM, Gao P, Cantor TL, et al. Improved assessment of bone turnover by the PTH-(1-84)/large C-PTH fragments ratio in ESRD patients. *Kidney Int* 2001;60:1460-8.
- Takasu H, Baba H, Inomata N, Ichyama Y, Kubota N, Kumaki K, et al. The 69-84 amino acid region of the parathyroid hormone molecule is essential for the interaction of the hormone with the binding sites with carboxyl-terminal specificity. *Endocrinology* 1996;137:5537-43.